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KS 327 (2012) (English): Edible Oils and Fats - Methods of sampling (Draft Standard)

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PUBLIC REVIEW DRAFT

Edible Oils and Fats - Methods of sampling and test

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Third Edition 2012

TECHNICAL COMMITTEE REPRESENTATION

The following organizations were represented on the Technical Committee:

University of Nairobi — Department of Food Science and Technology
Egerton University – Department of Human Nutrition
Jomo Kenyatta University of Science & Technology – Department of Food Science
Ministry of Public Health and Sanitation — Division of Food safety
— Division of Nutrition
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Government Chemist Department
Kenyatta National Hospital
Consumer Information Network
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Bidco Oil Refineries Ltd.
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Kenya Bureau of Standards — Secretariat

Revision of Kenya Standards

In order to keep abreast of progress in industry, Kenya Standards shall be regularly reviewed. Suggestions for improvements to published standards, addressed to the Managing Director, Kenya Bureau of Standards, are welcome.

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Edible Oils and Fats - Methods of sampling and test

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Foreword

This Draft Kenya Standard was revised by the Technical Committee on Edible Fats and Oils under the supervision of the Standards Projects Committee and is in accordance with the Procedures of the Bureau.

The standard provides the methods of test for parameters specified in the various product specifications

The revision was done to align the methods of test to the international test methods as provided for in the ISO standards. During the review and revision the following methods were deleted and replaced with ISO standards of similar scope and principles as they were obsolete. They include:
Determination of acid value;
Determination of peroxide value;
Determination of Iodine value;
Determination of matter volatile at 105 °C;
Determination of melting point (Slip Point) of solid fats; and
Method for determination of impurities in Edible oils and Fats

This third edition therefore cancels and replaces the second edition of the standard.

PUBLIC REVIEW DRAFT

EDIBLE OILS AND FATS — METHODS OF SAMPLING AND TEST

1 Scope

This standard prescribes the methods of sampling and test for refined edible oils and fats ready for human consumption.

2 Sampling

2.1 A container picked at random shall constitute a sample.

2.1.2 From each sample, draw a representative laboratory sample by cutting through diagonally across the surface so that the edges are also included for fats. For liquids, stir and take a sample.

2.1.3 Heat the laboratory sample in order to soften it in a container immersed in a water bath, the temperature of the bath shall be as low as possible and shall not exceed 10 °C above the melting point of produce. Excess heating shall be avoided because it may cause visible separation of the emulsion. Mix while heating in order to undo any separation that has started and to obtain uniformity of fluidity.

2.1.4 Remove the container from the bath, mix well (this can also be done by a mechanical mixer) until the specimen cools again and creamy consistency is obtained. Weigh Immediately a required test quantity.

2.2 Oils — A container sampled shall be mixed by thorough shaking.

2.3 Semi-solids — If the semi-solid sample is partially molten, then the whole container should be mixed by using a suitable mechanical agitator.

3 Determination of relative density *t*/20 °C

3.1 Principle — The relative density at *t*/20°C of an oil or fat is the ratio of the mass in air of a given volume of the oil or at *t* °C to that of the same volume of water at 20 °C, the weighings being made with weights adjusted to balance weight in air.

3.2 Apparatus — Pycnometer.

3.3 Procedure — Calibrate a relative density bottle or pycnometer (of capacity of at least 25 mL) as follows:

Clean and dry the bottle and weigh it in a bath of water at 20 °C until it reaches that temperature. If a bottle is used, insert the stopper in such a way that the capillary, if complete filled with water, and then maintain it at 20 °C until no further alteration in volume occurs. Wipe the stopper. If a pycnometer is used, adjust the volume of liquid to the fixed mark. Remove the bottle or pycnometer from the bath, dry the outside, allow to stand for a short time and weigh.

Empty and dry the bottle or pycnometer. Fill it with the sample of oil or fat previously brought near to the temperature of *t* °C. Keep the bottle or pycnometer in a bath adjusted to *t* °C until it has acquired that temperature. If a bottle is used, insert the stopper in such a way that the capillary is completely filled with the oil or the fat and then maintain it at the temperature *t* °C until no further alteration in volume occurs. Wipe the stopper. If a psychomotor is used, adjust the volume to the fixed mark. Remove the apparatus from the bath, dry the outside, allow to stand for a short time and weigh. Make all weighing air with weights adjusted to balance brass weights in air.

3.4 Calculation and Expression of Results

$$\text{Relative density} \pm /20\ ^\circ\text{C in air} = \frac{M_2}{M_1\,(1+\infty\ (t-20\ ^\circ\text{C}}$$

where,

m_2 = mass, in grammes, of oil or fat obtained in the test;

m_1 = mass, in grammes, of water obtained in calibration test; and

∞ = the coefficient of cubic expansion of glass at the given temperature.

∞ = 0.000 03 for soda glass.

∞ = 0.000 01 for borosilicale glass.

4 Determination of soap content

4.1 Principle — Volumetric titration with hydrochloric acid using bromophenol blue as indicator.

4.2 Reagents — The reagents used shall be of recognized analytical reagent quality.

4.2.1 *Distilled Acetone* — Containing 2 per cent of added water.

4.2.2 *Hydrochloric Acid* — 0.01N, accurately standardized.

4.2.3 *Bromophenol Blue Indicator* — 1 per cent solution in 95 per cent (v/v) ethanol.

4.3 Apparatus

4.3.1 *Test Tubes* — Approximately 150 x 40 mm of borosilicate glass fitted with ground glass stoppers and flattened at their ends.

4.3.2 *Micro-burette* — 5 mL.

4.4 Procedure — Prepare the test solution by adding 0.5 of the bromophenol *blue indicator* to each 100 mL of the *aqueous acetone* just before use and titration with 0.01 N acid or alkali until it is just yellow in colour. Weigh 40 g of the oil or fat to be tested into the test tube, which shall have been previously well rinsed with the test solution. Add 1 mL of warm water on the steam bath and shake vigorously. Add 50 mL of the neutralized aqueous acetone and after warming on the steam bath, shake the vessel well and allow the contents to stand until they separate into two layers. If soap is present in the oil or fat, the upper layer will be coloured green to blue.

Then add 0.01N acid, preferably from a micro-burette, until the yellow colour is restored. Continue the process of warming and shaking until the yellow colour of the upper layer remains permanent.

NOTE: It is convenient, but not essential, to run, at the same time, a blank on a soap-free oil. Any difference in colour between the upper layers can then readily be perceived.

4.5 Calculation and Expression of Results

Dissolved soap, as sodium oleate, per cent by mass

$$= \frac{0.304\,T}{m}$$

where,

T = volume, in mL, of 0.01N acid required, and

m = mass, in g, of sample taken.

Results are expressed as per cent m/m sodium oleate.

Note:
The above method is suitable for the determination of soap in oils up to 0.05 per cent. At higher concentrations, it is better to take 4 g of oil and use 0.01 N acid.

5. Determination of saponification value

5.1 Principle — Boiling of a sample under reflux with ethanolic potassium hydroxide solution, followed by titration of the excess potassium hydroxide with standard volumetric hydrochloric acid solution.

5.2 Reagents — All reagents shall be of recognized analytical grade and the water used shall be distilled water or water of equivalent purity.

5.2.1 *Potassium Hydroxide 0.5 N Aqueous Solution in 95 per cent (v/v) Ethanol* — This solution shall be colourless or straw yellow. A stable colourless solution can be prepared by either of the following procedures:

(*a*) Reflux 1 litre of ethanol with 8 g of potassium hydroxide and 5 g of aluminium pellets for 1 h, then distill immediately. Dissolve the required amount of potassium hydroxide in the distillate. Allow to stand for several days, then decant the clear supernatant liquid from the potassium carbonate deposited.

(*b*) Add 4 g of aluminium tertbutylate to 1 Litre of ethanol. Allow the mixture to stand for several days. Decant the supernatant liquid and dissolve in it the required amount of potassium hydroxide. Allow to stand for several days, then decant the clear supernatant liquid from the potassium carbonate deposited.

Store this solution in a brown or yellow glass bottle fitted with a rubber stopper, and decant it for use.

5.2.2 *Hydrochloric Acid, 0.5 N Standards Volumetric Solution*

5.2.3 Phenolphthalein 10 g/L solution in 95 per cent (v/v) ethanol, or alkali blue 68, 20 g/L solution in 95 per cent (v/v) ethanol.

5.2.5 *Boiling Aids*

5.3 **Apparatus —** Usual laboratory apparatus and, in particular, the following:

5.3.1 *Conical Flask* **—** Of 250 mL capacity, made of alkali-resistant glass and having a ground neck.

5.3.2 *Reflux Condenser* **—** With a ground glass joint, which fits the conical flask (**8.3.1**).

5.3.3 *Heating Device* (*e.g. a Water-Bath, Electric Hot-Plate or other Suitable Apparatus)* — A naked flame is not suitable.

5.3.4 *Burette* — Of 50 mL capacity, graduated in 0.1 mL divisions.

5.3.5 *Pipette* — Of 25 mL capacity

5.4 Procedure

5.4.1 **Test Portion —** Weigh, to the nearest 5 mg, about 2 g of the test sample into a conical flask (**8.3.1**).

NOTE: The test portion of 2 g has been determined on the basis of saponification values of 170 to 200. For other saponification values, the mass should be altered accordingly so that about half the ethanolic potassium hydroxide solution is neutralized.

5.4.2 Determination

5.4.2.1 Using a pipette (**8.3.5**), add to the test portion 250 mL of the ethanolic potassium hydroxide solution (**8.2.1**) and some boiling aids (**8.2.4**). Connect the reflux condenser (**8.3.2**) to the flask, place the flask on the heating device (**8.3.3**) and boil gently, shaking from time to time, for 60 min or for 2 h in the case of oils and fats having a high melting point and which are difficult to saponify.

5.4.2.2 Add to the hot solution 0.5 mL to 1 mL of the phenolphthalein solution (**8.3.3**) and titrate with the standard volumetric hydrochloric acid solution (**8.2.2**) until the pink colour of the indicator just disappears. If the solution is strongly coloured, use 0.5 mL to 1 mL of alkali blue 68 solution (**8.2.3**).

5.4.3 **Blank Test —** Carry out a blank test following the procedure specified in (**8.4.2.1**) using again 250 mL of the ethanolic potassium hydroxide solution (**8.2.1**) but omitting the test portion.

5.4.4 *Number of Determinations* — Carry out two determinations on the same test sample.

5.5 **Expression of Results —** The saponification value I_s is given by the formula:

$$I_s = \frac{(v_0 - v_1)}{m} \times c \times 56.1$$

where,

v_o = the volume, in millilitres, of the standard volumetric hydrochloric acid solution (**8.2.2**) used for the blank test;

v_1 = the volume, in millilitres, of the standard volumetric hydrochloric acid solution (**8.2.2**) used for the determination;

c = the exact concentration, in moles per litre, of the standard volumetric hydrochloric acid solution (**8.2.2**);

m = the mass, in grams, of the test portion (**8.4.1**)

6 Determination of unsaponifiable matter

6.1 **Principle —** Saponification of the fat or oil by boiling under reflux with an ethanolic potassium hydroxide solution. Extraction of the unsaponifiable matter from the soap solution by diethyl ether. Evaporation of the solvent and weighing of the residue after drying.

6.2 **Reagents —** All reagents shall be of recognized analytical grade. The water used shall be distilled water or water of at least equivalent purity.

6.2.1 *Diethyl Ether* — Freshly distilled, free from peroxides and residue.

6.2.2 *Acetone*

6.2.3 *Potassium Hydroxide* — 1N ethanolic solution.

Dissolve 60 g of potassium hydroxide in 50 mL of water and dilute to 1000 mL with 95 per cent (v/v) ethanol. The solution shall be colourless or straw-yellow.

6.2.4 *Potassium Hydroxide* — 0.5N acqueous solution.

6.2.5 *Phenolphthalein* — 10 g/L solution in 95 per cent (v/v) ethanol*.*

6.3 **Apparatus —** Usual laboratory apparatus and, in particular, the following:

6.3.1 *Round-bottomed Flasks* — Of 250 mL capacity, with ground neck.

6.3.2 *Reflux Condenser* — With ground joint to fit the flasks (**8.3.1**)

6.3.3 *Separating Funnels* — Of 500 mL capacity, with stopcock and stopper made of polytetrafluothylene*.*

6.3.4 *Boiling Water Bath*.

6.3.5 *Oven* — Capable of being maintained at 103 ºC ± 2 ºC.

6.4 Procedure

6.4.1 *Test Portion* — Weigh, to the nearest 0.01 g, about 5 g of the test sample into 250 mL flask (**9.3.1**).

6.4.2 *Saponification* **—** Add 50 mL of the potassium hydroxide solution (**9.2.3**) and a few anti-bumping granules. Attach the reflux condenser (**9.3.2**) to the flask and boil the contents gently for 1h. Stop heating. Add 100 mL of water through the top of the condenser and swirl.

NOTE: If the extraction of the unsaponifiable matter is carried out with a view to the determination of the composition of the tocopherols, the addition of phyrogallol is necessary and the extraction should be completed quickly (say, within 30 min).

6.4.3 *Extraction of the Unsaponifiable Matter* **—** After cooling, transfer the solution to a 500-mL separating funnel (**9.3.3**). Rinse the flask and the anti-bumping granules several times with the diethyl ether (**9.2.1**), using 100 mL, and pour these rinsings into separating funnel. Stopper and shake vigorously for 1 min, periodically releasing pressure by inverting the separating funnel and cautiously opening the stopcock.

Allow to stand until there is complete separation of the two phases. Then run off the lower layer as completely as possible into a second separating funnel.

NOTE: If an emulsion is formed, destroy it by adding small quantities of ethanol or concentrated potassium hydroxide or sodium chloride solution.

Extract the aqueous ethanolic soap solution twice more, each time in the same way with 100 mL of the diethyl ether. Collect the three ether extracts in one separating funnel containing 40 mL of water.

6.4.4 *Washing of the Ethereal Extract* — Gently rotate the separating funnel containing the combined extracts and the 40 mL of water.

NOTE: Violent shaking at this stage may result in emulsions.

Allow the layers to separate completely and draw off the lower aqueous layer. Wash the ethereal solution twice more with 40-mL portions of water, shaking vigorously each time and discarding the lower aqueous layer after separation. Draw off each washing solution leaving 2 mL, then rotate the separating funnel around its axis. Wait some minutes to allow the remaining acqueous layer to collect. Draw this off, closing the stopcock when the ethereal solution reaches the bore of the stopcock.

Wash the ethereal solution successively with 40 mL of the potassium hydroxide solution (**9.2.4**), 40 mL of water, and again with 40 mL of potassium hydroxide solution, then at least twice more with 40 mL of water.

Continue to wash with water until the washings no longer give a pink colour on the addition of a drop of the phenolphthalein solution (**9.2.5**).

6.4.5 *Evaporation of the Solvent* **—** Transfer the ethereal solution quantitatively, little at a time, through the top of the separating funnel into a 250-mL flask (**9.3.1**) previously dried at 103 ºC ± 2 ºC in the oven (**9.3.5**), cooled and weighed to the nearest 0.1mg. Evaporate the solvent on a boiling water-bath (**9.3.4**).

Add 5 mL of acetone (**9.2.2**) and evaporate the volatile solvent completely in a gentle current of air, holding the flask obliquely while turning it in a boiling water-bath.

6.4.6 *Drying the Residue and Determination* **—** Dry the residue in the oven (**9.3.5**) at 103 ºC ± 2 ºC for 15 min, with the flask in an almost horizontal position. Allow to cool in a desiccator and weigh to the nearest 0.1 mg.

Repeat the drying for successive 15 min periods until the loss of mass between two successive weighings is less than 1.5 mg. If a constant mass is not obtained after three periods of drying, the unsaponifiable matter is probably contaminated and the determination shall be repeated.

NOTE:

1. If available, a vacuum rotary evaporator may be used, particularly if the unsaponifiable matter is to be examined further.

2. If a correction for free fatty acids is considered necessary after weighing the residue, dissolve it in 4 mL of the diethyl ether (**9.2.1**) and then add 20 mL of ethanol previously neutralized to a faint pink colour in the presence of the phenolphthalein (**9.2.4**) as indicator. Titrate with standard volumetric ethanolic potassium hydroxide solution c, (KOH) = 0.1 mol/L, to the same final colour. Calculate the mass of free fatty acids as oleic acid and correct the mass of the residue accordingly (*see* **9.5**).

6.4.7 *Number of Determinations* **—** Carry out two determinations on the same test sample.

6.4.8 *Blank Test* **—** Carry out a blank test, using the same procedure and the same quantities of all the reagents, but omitting the test portion. If the residue exceeds 1.5 mg, investigate the technique and the reagents.

6.5 Expression of Results — The unsaponifiable matter content, expressed as a percentage by mass of the sample, is equal to

$$\frac{100\left(m_1 - m_2 - m_3\right)}{m_0}$$

where,

m_o = the mass, in grams, of the test portion;

m_1 = the mass, in grams, of the residue;

m_2 = the mass, in grams, of the residue;

m_3 = the mass, in grams, of free fatty acids, if any (*see* **9.4.6,** Note 2) and equals 0.28 V_c

where,

V = the volume, in millilitres, of the standard volumetric ethanolic potassium hydroxide solution used for titration.

c = the exact concentration, in moles per litre, of the standard volumetric ethanolic potassium hydroxide solution.

Take as the result the arithmetic mean of the two determinations.

7 Determination of metals other than arsenic and mercury (*atomic absorption method*)

7.1 Preparation of Sample

7.1.1 Weigh sample containing 20 g or less depending upon expected copper content. If sample contains less than 75 per cent H_2O, add H_2O to obtain this dilution. Add initial volume HNO_3 to equal about twice dry sample weight and 5 mL H_2SO_4, or as much H_2SO_4 as g dry sample, but less or equal 5 mL.

7.1.2 Place the weighed sample in 800 mL or any other suitable capacity pyrex kjeldahl flask. Add 25 mL to 58 mL HNO_3, then cautiously add 20 mL H_2SO_4. Place each flask on asbestos mat with 5.1 cm diameter hole. Warm slightly and discontinue heating if foaming becomes excessive.

7.1.3 When reaction has quieted, heat flask cautiously and rotate occasionally to prevent caking of sample upon glass exposed to flame. Maintain oxidizing conditions in flask at all times during digestion by cautiously adding small amounts of HNO_3 whenever mixture turns brown or darkens. Continue digestion until organic matter is destroyed and SO_3 fumes are copiously evolved. (Final solution should be colourless, or at most light straw colour). Cool slightly and add 75 mL H_2O and 25 mL saturated NH_4 – Oxalate to assist in expelling oxides of nitrogen from solution. Evaporate again to point where fumes of SO_3 appear in the neck of flask. Cool, and dilute with H_2O to 500 or 1000 mL in volumetric flask.

NOTE: As the sample contains large amount of fat, partial digestion is carried out with HNO_3 until only fat is undissolved. Cool, filter free of solid fat. Wash residue with H_2O, add H_2SO_4 to filtrate, and complete digestion as above. After digestion, cool, add 25 mL H_2O, and remove nitrosyl sulphuric acid by heating to fumes. Repeat adding 25 mL H_2O and fuming. If after cooling and diluting, insoluble matter is present, filter through acid washed paper, rinse paper with H_2O, and dilute to 100 mL.

7.1.4 *Determination of Reagent Blank* — Carry out digestion of the reagents without samples.

7.1.5 *Preparation of Standards*

7.1.5.1 *Stock solutions* — Stock solution of purifical metals or their salts are stored in polythene bottles at a concentration of 1.0 mg/mL, in preparing these solutions, exact weights must be taken to ensure accuracy of these standards and, hence, the sample estimations.

7.1.5.2 *Working standards (usually 0.5 and 1.0 mcg/mL*) **—** These are freshly prepared each day. All flasks and pipettes used in the preparation of the working standards (0.5 and 1.0 mcg/mL) must be acid washed and rinsed in distilled water. Acid used in the preparation of solutions must be “Foodstuff Analysis Grade”.

Pipette 1.0 mL of stock solution into a 1-litre volumetric flask and dilute to volume with distilled water (several different standards may be pipetted into the same flask as long as chemical reactions do not occur). 5.0 mL and 10.0 mL aliquots of the diluted solution are then pipetted into 100 mL distilled water to give 0.5 mcg/mL and 1.0 meg/mL working standards.

NOTES:

1. If the sample solutions are prepared using hydrochloric acid, then 1.0 mL of concentrated hydrochloric acid must be added to each working standard before dilution.
2. If the sample contain sodium ions or sodium chloride, then 1.0 mL of a 10 per cent solution of analar sodium chloride must be added to each working standard before dilution.

7.1.6 *Determination* **—** Determine trace metals by first aspirating the standards the value of whose readings are utilized in a drawing standard curve. Then aspirate the sample solutions.

NOTE: The instructions of the manufactures of the atomic absorption instrument must be strictly followed.

7.1.7 *Calculation* — From the reading of the sample solutions obtained on the calibration curve, calculate the concentrations of the trace metal in the original samples.

8 Determination of arsenic (*gutzeit method*)

8.1 Apparatus

8.1.1 *Generator and Absorption Tubes* — Use 56.70 g wide mouth bottles of uniform capacity and design as generators, and fit each by means of perforated stopper with glass tube 1 cm diameter and 6.7 cm long with additional constricted end to facilitate connection. Place small wad of glass wool in constricted bottom end tube and add 3.5 to 4 g sand, taking care to have some amount in each tube. Moisten sand with 10 per cent $Pb(CAC)_2$ solution and remove excess by suction. Clean and when necessary by treatment (do not remove sand from tube) with HNO_3 followed by H_2O rinse under suction. Treat with $Pb(CAC)_2$ solution. If sand has dried though, clean and remoisten it as directed. Connect tube by means of rubber stopper with narrow glass tube 2.6 mm to 2.7 mm internal diameter and 10 cm to 12 cm long, and introduce clean end of strip of $HgBr_2$ paper. (3 mm bore allows strip to curl, which results in uneven strain and poor point). Clean and dry tube before inserting $HgBr_2$ paper. (Ordinary pipe cleaner may be used.)

8.1.2 **Water Bath —** Use constant temperature bath. If no water is available, use flat-bottom container of suitable depth and capacity. (A deep water bath is suggested to ensure uniform conditions during evaluation and absorption of the AsH_3).

8.2 Reagents

8.2.1 *Stannous Chloride Solution* — Dissolve 33 g of stannous chloride in 10 mL of conc. hydrochloric acid and dilute to 100 mL of water. Add 100 mL of concentrated hydrochloric acid, boil down to 100 mL and filter.

8.2.2 *Zinc* — Use 20 mesh or 30 mesh, arsenic-free granulate zinc either cut into 1 cm lengths or melted and cast into pellets in porcelain mold drilled (for example) 9 mm diameter and 12.5 mm deep. Activate the pieces of zinc with HCl (1+3), containing 2 mL $SnCl_2$ solution 100 mL, letting action continue for 15 min. Discard distinctly inactive or over active pieces and pour off liquid. Wash zinc free from acid with clear tap water and rinse with hot H_2O. Select uniformly etched non-pitted zinc and store in suitable receptacle. Maintain supply of uniform zinc with raw zinc from original receptable until stock is exhausted and store used zinc in second receptacle after discarding non-uniform or deeply pitted pieces. Draw zinc from second receptable after washing it with running water. Repeat procedure until pieces are too small for further use.

8.2.3 *Potassium Iodide Solution* **—** Dissolve 15 g KI in H_2O and dilute to 100 mL.

8.2.4 *Sand* **—** Clean 30-mesh (through No. 30 but not No. 40) White Sea sand by washing successively with hot 10 per cent NaOH solution, hot HNO_3, and hot H_2O. Dry the clean sand.

8.2.5 *Mercuric Bromide Paper* **—** Use whatman No. 40 or its equivalent and cut paper of uniform weight and texture into strips exactly 2.5 mm wide and about 12 cm long. (Uniformity in width and texture of paper is of great importance. Irregular texture produces irregular impregnation with consequent inaccurate results). To sensitize, soak strips in 3 to 6 per cent (optimum 5 per cent) filtered solution of $HgBr_2$ in alcohol, for 1 h or longer according to amount, character, and activity of zinc used. (Attenuated, unsatisfactory stains, caused by over-rapid evaluation of arsenic H_3, can be shortened and intensified by increasing concentration of $HgBr_2$ and vice versa). If strips are in sheets, cut off 2 sides before soaking and leave strips attached at ends. After sensitization, remove strips and dry individual ones on glass rods; dry strips by waving them in air.

Place strips when nearly dry between clean sheets of paper and subject to pressure long enough to take out bends or curls. Store in dry, dark place. (Aging or impregnated strips usually results in markedly fainter and long strains. Desirable types of strain result from use of impregnated strips 2 days old). When ready for use, cut individual strips off squarely 12.7 mm from one end and insert this end into narrow tube apparatus. Handle sheets by paper attached to either end and cut in half just before use. Strips must be clean and free from any contamination.

8.2.6 *Arsenic Standard Solution*

8.2.6.1 *Stock solution* — 0.132 g arsenic trioxide, 50 mL conc. hydrochloric acid and dilute to 100 mL with water.

8.2.6.2 *Intermediate solution* — 0.04 mg As_2O_3/mL. Dilute 40 mL stock solution to 1 litre.

8.2.6.3 *Working solution* — 0.002 As_2O_3/mL. Dilute 50 mL intermediate solution to 1 litre and use to prepare standard strains. Solution containing 0.000 1 mg. As_2O_3 may also be prepared.

8.3 **Preparation of Sample —** Weigh 5 g to 50 g according to moisture content and quantity of arsenic expected and place in Kjeldahl flask of appropriate capacity. Add 25 to 50 mL HNO_3; then cautiously add 20 mL H_2SO_4. Place each flask on asbestos mat with 5.1 cm diameter hole. Warm slightly and discontinue heating if foaming becomes excessive. When reaction has quieted, heat flask cautiously and rotate occasionally to prevent caking of sample upon glass exposed to flame. Maintain oxidizing conditions in flask at all times during digestion by cautiously adding small amounts of HNO_3 whenever mixture turns brown or darkens. Continue digestion until organic matter is destroyed and SO_3 fumes are copiously evolved. (Final solution should be colourless or at most light straw colour.) Cool slightly, and add 75 mL H_2O and 25 mL saturated NH_4 oxalate solution to assist in expelling oxides of nitrogen from solution. Evaporate again to point where fumes of SO_3 appear in neck of flask. Cool, and dilute with water to 500 mL or 100 mL in volumetric flask.

8.4 **Isolation of Arsenic —** Before making determinations, isolate As, when interfering substances are present in digestions (e.g. pyridine from tobacco), or when samples contain excessive amounts of salts, or H_2SO_4 from digestions. After cooling, add 7 mL of water, 5 g of the chloride hydrazine- bromide mixture (avoiding contamination of the neck of the flask) and 100 mL of conc. hydrochloric acid. Fit the condenser on the flask immediately and distil into an externally cooled mixture of 2-mL concentrated nitric acid and 10 mL of water.

The exit end of the condenser must dip below the surface of the liquid. Continue distilling until the liquid in the Kjeldah flask is reduced to about half or until 5 min after the condenser is full of steam. Evaporate the distillate to dryness in a 100-mL beaker on the water bath and then evaporate twice more after the addition of 5 ml of water to remove the nitric acid. Dissolve the residue to a suitable volume. The solution can then be used for the specific estimation of arsenic by the Gutzeit method, the molybdenum-blue procedure or by the silver diethyldthiocarbanite method.

8.5 Determination

8.5.1 Determine acid (HCl or H_2SO_4) according to previous treatment by titrating, if necessary, in definite volume of sample solution. Place aliquots containing 0.01 mg to 0.03 mg As_2O_3 (0.020 mg to 025 mg is optimum), and 30 mL, in Gutzeit generator. If the liquor taken is found to be outside limits specified, repeat with proper aliquot. If aliquot contains only HCl, add enough HCL to make total volume of 5 mL if it contains H_2SO_4, add enough 25 per cent arsenic-free NaOH solution (keep in arsenic-free pyrex) to exactly neutralize it and add 5 mL HCl, or add enough HCl to the H_2SO_4 in aliquot to make total volume of 5 mL mixed acids. Cool when necessary and add 5 mL KI reagent and 4 drops $SnCl_2$ solution (**12.2.1**).

8.5.2 Prepare standards corresponding to 0.010 mg, 0.020 mg and 0.030 mg As_2O_3 from arsenic working standard solution (**12.6.3**). Since standards must contain same kind and amounts of acid sample, add 5 mL HCl, or H_2SO_4, and HCl (total 5 mL), according to prior treatment of unknown. If H_2SO_4 has been neutralized, add equivalent amount of arsenic-free Na_2SO_4 to standards. Mix and let it stand for 30 min at 25 ºC or 5 min at 90 ºC. Dilute with H_2O to 40 mL.

8.5.3 Prepare generator as in **12.1.1** and carefully centre strip of HgB_{r2} paper in narrow tube. If sheets of strips are used, prepare sample and standard strips from same strip group. According to activity of zinc, add to each standard and sample 10 g to 15 g activated stick zinc or 2 g to 5 g granulated zinc; add same amount to each generator.

Equalize, as far as possible, the surface area of zinc exposed in standard and sample.

8.5.4 Immerse apparatus to within 2.5 cm of top of narrow tube in H_2O bath kept at temperature of 20 °C to 25 º C, and let evaluation process continue for 1h to 5 h. Remove strip and average standard strips on cross-section paper, using length in mm as ordinate and mg As_2O_3 as abscissa. Locate length of unknown strip on standard graph and read off an abscissa quantity of As present. Report only to third decimal as grains As_2O_3/1*b*.

8.6 **Calculation —** Take smaller or larger aliquots when stain is longer or shorter than highest or lowest standard respectively.

Grains/1*b* x 143 = ppm

ppm x 0.007 = grains/1*b*

Performance blanks frequently. Blanks should not show more than 0.001 mg As_2O_3.

9. Determination of iron content

9.1 **Principle —** This method is considered satisfactory for the determination of iron occurring normally in commercial oils and fats, i.e. as scaps or in their organically combined forms in solution in the oil. Acid extraction suffices for these cases and has been found preferable to the ashing technique. Where, however, the presence of iron form extraneous sources in less soluble inorganic forms, e.g. ferrosilicate, is suspected, total iron should be determined by ashing and alkali fusion. The pink colour formed by reaction of Fe+++ with thioglycollic acid.

9.2 **Reagents —** The reagents used shall be of recognized analytical reagent quality and free from iron. Distilled water, re-distilled from all-glass apparatus, shall be used throughout.

9.2.1 *Hydrochloric* Acid, *d* = 1.18

9.2.2 *Ammonia* Solution, *d* = 0.880

9.2.3 *Thioglycollic acid*

9.2.4 *Bromine water saturated*

9.2.5 *Standard Iron Solution* — Dissolve 8.635 g of ammonium ferric sulphate, $NH_4Fe(SO_4).12H_2O$ in distilled water containing 5 mL of dilute (1:3) sulphuric acid and dilute to 1000 mL. Before use, dilute this stock solution one hundred times by successive dilutions. 1 mL of the resulting solution is equivalent to 10 microgrammes Fe.

9.3 Apparatus

9.3.1 *Flask* — Of 250 mL capacity, with interchangeable conical ground glass joints.

9.3.2 *Spectrophotometer or Photoelectric Absorptiometer* — With a blue-green filter having a maximum transmission at approximately 480 mm; all glass cells should be used, of such size (1 cm cells are usually satisfactory) that the optical density of the solution under test lies between 0.1 and 0.8.

9.3.3 *Nessler Cylinders* — Of 50-mL capacity.

9.4 **Procedure —** Weigh 25 g of the oil or fat into the flask. Add 15 mL of water and 20 mL of the hydrochloric acid. Use a few glass beads to regulate the boiling. Reflux for one hour. Transfer to a separating funnel, allow settling and running off the aqueous layer through a double, acid-washed medium texture filter paper into a 300-mL beaker (a whatman No. 40 or Barcham Green No. 803 paper or equivalent, washed with hydrochloric acid of the same concentration as that used for the extraction immediately before use, is suitable). Wash the oil or the fat again in the separating funnel. With 50 mL portions of hot water, using these to rinse the flask and pouring them through the filter after washing the oil or the fat. Evaporate the combined aqueous and acid extracts to about 5 mL and add 5 mL of bromine water.

Boil gently until the excess bromine is driven off. When cool, transfer to the Nessler cylinder. Add 2 to 3 drops of thioglycollic acid, make just alkaline to litmus by the dropwise addition of ammonia solution and dilute to 50 mL with water. Read the colour in the absorptiometer or in the spectrophotometer.

9.5 **Calculation and Expression of Results —** Determine the iron content of the solution from a standard curve prepared as follows:

Measure into five separate 50 mL graduated flasks by means of a burette, 0 mL, 5 mL, 10 mL, 15 and 25 mL of the standard iron solution. Add 5 mL of water, 3 mL of the hydrochloric acid, 2 or 3 drops of thioglycollic acid and ammonia solution dropwise until the solutions are just alkaline to litmus paper. Cool, dilute to the mark with the distilled water and mix well. Determine the colours of the solutions in the appropriate instrument, using in the reference cell the control solution containing no iron. Plot a curve correlating transmission or optical density against micro-grammes of iron.

Alternatively, the iron content may be determined by matching with standards in Nessler cylinders, as follows:

Place in a 50-mL Nessler cylinder, 35 mL of water, 3 mL of the hydrochloric acid and 2 to 3 drops of thioglycollic acid and ammonia solution until the solution is just alkaline to litmus paper. Cool, dilute to 50 mL with water and add from a burette, with constant stirring, standard iron solution until the colour of the solution matches that of the test solution. Note the volume of standard iron solution used. Repeat the preparation of the maching solution, but place the volume of standard iron solution previously determined in the Nessler tube before adding the reagents. If the standard colour is now different from that of the test solution, prepare other standards containing appropriately more or less iron until a perfect match is obtained. The best visual matches are obtained when the solution in the Nessler cylinder contains 1 mL to 2 mL of standard iron solution. Results are expressed as mg iron/kg oil or fat.

10. Determination of allyl isothiocyanate content

10.1 Principle — The oil obtained from black mustard seeds contains sinigrin and myrosin, which, after maceration with water, yields a volatile oil, the major constituent of which is allyl isothiocyanate. The oil obtained from mustard seeds contains acrinyl isothiocyanate, which is much less volatile than acrinyl isothiocyanate

The allyl isothioyanate in the oil is steam distilled into a known excess of silver nitrate solution, and the excess of silver nitrate solution is determined by titration with standard ammonium thiocyanate solution.

10.2 Reagents — The reagents used shall be of recognized analytical reagent quality.

10.2.1 *Ethanol* — 95 per cent v/v, neutral to phonophtalein.

10.2.2 *Silver Nitrate Solution* — Approximately 0.1 N.

10.2.3 *Ammonium Hydroxide Solution 10% (m/v)*

15.2.4 *Nitric Acid* — 69 per cent m/m HNO_3.

10.2.5 *Ferric Ammonium Sulphate Indicator* — 0.1 per cent (m/v) solution in water.

10.2.6 *Standard Ammonium Thiocyanate Solution* — Approximately 0.1N.

10.3 Apparatus

10.3.1 *Distillation Flask* — 500-mL round bottomed flask.

10.3.2 *Any Efficient Reflux Condenser* — Approximately 900 mm long.
10.3.3 *Measuring Flask* — 200-mL capacity

10.3.4 *Water Bath*

10.4 Procedure — Weigh accurately approximately 5 g of the material into a 500-mL distillation flask and add to it 25 mL of ethanol 250 mL of water and a few pieces of pumice stone. Distill the mixture in steam and collect the distillate in a 200 mL measuring flask containing exactly 25 mL of silver nitrate solution and 10 mL of ammonium hydroxide solution. Collect as rapidly as possible about 150 mL of the distillate. Attach the reflux air condenser to the measuring flask and heat the mixture for about one hour on a boiling water bath. Cool to room temperature and make up to 200 mL and filter the content after shaking. Take 100 mL of the filtrate, add 6 mL nitric acid and a few drops of ferric ammonium filtrate, add 6mL of nitric acid and few of ferric ammonium sulphate indicator, and titrate with standard ammonium thiocyanate solution until a permanent red colour is obtained. Carry out a blank test at the same time.

10.5 Calculation and Expression of Results

$$\text{Allyl isothiocyanate, g/kg} = \frac{9.915\,(v_o - v_1)\,N \times 10}{m}$$

where,

V_0 = volume, in mL of standard ammonium thiocyanate solution required for the blank determination;

V_1 = volume in mL of standard ammonium thiocyanate solution required for the sample;

N = normality of standard ammonium thiocyanate solution; and

m = mass in g of the sample taken for the test.

11. Determination of carotene content

11.1 Definition — The carotene of palm oil is defined and calculated as B-carotene in parts per million (ppm).

11.2 Principle — Spectrophotometric measurement at 446 nm of the absorbance of a homogenized and diluted sample.

11.3 Reagent

11.3.1 *Trimethylpentane (ISO-octane) or n-hexane* — Optically pure at 446 nm.

11.4 Apparatus

11.4.1 *Spectrophotometer* — With 1 cm quartz cuvuttes suitable for measurement at 446 nm.

11.4.2 *Volumetric Flask* — 25 mL capacity.

11.4.3 *Pipette — 5 mL*

11.5 Preparation of Sample — Melt the sample at 60 ºC to 70 ºC and homogenize thoroughly before taking a test portion. Filter through a fast filter paper if the sample contains impurities or is not clear.

11.6 Procedure — Weigh, to the nearest 0.0001g, 0.3 g of the sample into the 25 mL volumetric flask. Dissolve the test portion with a few millilitres of solvent and dilute to the mark.

Pipette accurately 5 mL of the prepared solution into another 25 mL volumetric flask and make up to volume with the same solvent.

Transfer the diluted solution to the 1 cm cuvette and measure the absorbance at 466 nm against the solvent used. Correct for cuvette error at the same wavelength.

11.7 Expression of Results — The carotene content is expressed as ppm B-carotene and is given by

$$\frac{478.75\left(a_S - a_b\right)}{w}$$

where,

a_s = the absorbance of the sample;

a_b = the cuvette error;

w = the weight of sample in grammes.

Express the results to the nearest unit

12. Preparation of methyl esters of fatty acids for gas-liquid chromatography analysis

12.1 **Definition —** This standard describes methods of preparing the methyl esters of fatty acids from palm oil products.

12.2 General Method Using Borom Trifluoride

12.2.1 *Principle* **—** Saponification of the glycerides and esterification of the liberated fatty acids in the presence of boron trifluoride.

12.2.2 *Reagents*

12.2.2.1 *Sodium hydroxide, methanolic solution, approximately 0.5 N* — Dissolve 2 g of sodium hydroxide in 100 mL of methanol containing not more than 0.5 per cent m/m of water. If the solution has to be stored for a considerable time, a small amount of white precipitate of sodium carbonate may be found. This has no effect on the preparation of the methyl esters.

12.2.2.2 Boron trifluoride methanolic solution, 12 per cent to 15 per cent w/v available commercially or may be prepared in the laboratory (*see* Note 1).

12.2.2.3 *Heptane* — Of chromatographic quality.

12.2.2.4 *Petroleum ether* — Redistilled (boiling range 40 ºC to 60 ºC) or n-hexane.

12.2.2.5 *Sodium sulphate* — Anhydrous
12.2.2.6 *Sodium chloride* — Saturated aqueous solution.

12.2.2.7 *Methyl red* — 0.1 per cent in 60 per cent v/v ethanol.

12.2.2.8 *Nitrogen* — High purity.

12.2.3 *Apparatus*

12.2.3.1 *Flask* — 50 mL or 100 mL with ground neck.

12.2.3.2 *Reflux condenser* — 20 cm to 30 cm effective length, with ground joint to fit the flask.

12.2.3.3 *Boiling aid* — Fat free.

12.2.3.4 *Graduated pipette* — Capacity at least 10 mL.

12.2.3.5 *Test-tube* — With ground neck and fitted with a ground glass stopper or a glass doa; fitted with a tef;pm-lined cap.

12.2.3.6 *Separating funnels* — 250 mL.

12.2.4 *Preparation of Sample* — Melt the sample at 60 ºC to 70 ºC and homogenize thoroughly before taking a test portion.

12.2.4.1 *Procedure* **—** Weigh out roughly a test portion of 0.3 g into the 50 mL or 100-mL flask. Add 5 mL of the methanolic sodium hydroxide solution and a boiling aid. Attach a condenser and boil under reflux until the fat droplets disappear. This usually takes 5 min to 10 min.

Add 7 mL of the methanolic boron trifluoride solution from the graduated pipettes through the top of the condenser and continue boiling for two minutes. Add about 3 mL of heptane to the boiling solution through the top of the condenser and continue boiling for another one minute. Stop heating, cool to room temperature and then remove the condenser. Add a small portion of the saturated sodium chloride solution and swirl the flask gently several times. Add more saturated sodium chloride solution to bring the liquid level into the neck of the flask. Transfer about 1 mL of the heptane solution (upper layer) into a test-tube and add a small amount of anhydrous sodium sulphate. This solution may then be injected directly into a gas chromatograph.

To recover dry esters, transfer the saline solution and the heptane phase to a 250-mL separating funnel. Separate the layers and retain the heptane solution. Extract the saline solution twice with 50-mL portions of redistilled petroleum ether. Wash the combined extract with 20-mL portions of water until free from acid, using the methyl red solution as indicator. Dry over anhydrous sodium sulphate, filter and evaporate the solvent under a stream over nitrogen of a water-bath.

12.3 **Alternative Method using Potassium Methulate** (*Applicable to Palm Oil or Free Fatty Acid Content Below 5 Per Cent*).

12.3.1 *Principle* **—** Methanolysis of the glycerides in an alkaline medium.

12.3.2 *Reagents*

12.3.2.1 *Methanol* — Containing not more than 0.5 per cent v/v of water.

12.3.2.2 *Sodium methylate* — Approximately 0.5 N. Dissolve 1.2 g of sodium in 100-mL of the methanol. (*see* Note 2).

12.3.2.3 *Heptane of chromatographic quality*.

12.3.2.4 *Sodium sulphate* — Anhydrous.

12.3.2.5 *Nitrogen* — Of high purity.

12.3.3 *Apparatus*

12.3.3.1 *Water-bath*

12.3.3.2 *Flask* — 560 mL, with ground neck.

12.3.3.2 *Reflux condenser* — With ground joint to fit the flask.

12.3.3.4 *Boiling aids* — Fat free.

12.3.3.5 *Separating funnel* — 100 mL.

12.3.3.6 *Round-bottomed flask* — 100 mL with narrow neck.

12.3.4 *Preparation of sample* — Melt the sample at 60 ºC to 70 ºC and homogenize thoroughly before taking a test sample.

12.3.5 **Procedure —** Introduce approximately 0.3 g of the sample into the flask. Add about 20 mL of the sodium methylate solution and a boiling aid. Attach the condenser and bring to the boil. The solution should become homogeneous and clear. The reaction is normally complete after 5 min to 10 minutes.

Cool the flask and transfer the contents to a separating funnel. Rinse the flask into the separating funnel with 20 mL of the heptane. Add about 30 mL of water. Shake and allow to separate. Draw off the aqueous (bottom) phase into another 20 mL of heptane. Combine the heptane extracts and wash twice with 20 mL portions of water. Filter over anhydrous sodium sulphate into a 100-mL flask and evaporate the solution to 1 mL to 2 mL under a stream of nitrogen over a water-bath or using a rotary evaporator.

NOTE 2: Sodium should be carefully handled under paraffin or n-hexane when preparing the sodium methylate solution.

12.4 Special method for preparation of methyl esters of fatty acids with less than 12 carbon atoms.

12.4.1 *Principle* — Transesterification of the glycerides by reaction with methanolic potassium hydroxide solution.

12.4.2 *Reagents* **—** Unless stated otherwise, reagents and solvents shall be of analytical quality, and water shall be distilled water or water of equivalent quality.
12.4.2.1 *Potassium hydroxide* — Methanolic solution, approximately 2N. Dissolve 11.2 g of potassium hydroxide in 100 mL of methanol containing not more than 0.5 per cent v/v of water.
12.4.2.2 *Heptane* — Of chromatographic quality

12.4.3 *Apparatus* — Usual laboratory equipment, and in particular:

12.4.3.1 *Test-tube* — 20 mL with ground glass stopper.

12.4.3.2 *Volumetric flasks* — 50 mL and 100 mL.

12.4.3.3 *Graduated pipette* — Capacity mL or more.

12.4.3.4 *Measuring cylinder* — 10 mL.

12.4.4 *Preparation of the Test Sample* — The test sample shall be dry and clear. Melt at 50 ºC to 70 ºC and hamogenize before taking a post portion.

12.4.5 *Procedure* **—** Weigh, about 0.3 g of the test sample, in the test tube. Add to the test-tube 10 mL of heptane from the measuring cylinder.

Add 0.5 mL of the methanolic potassium hydroxide solution, stopper the test-tube and mix the contents by shaking until the solution becomes clear, this will take about 20 seconds. Just after this clarification, the solution becomes cloudy again as a result of the separation of the glycerol. Settling of the glycerol takes place rapidly. Decant the upper layer, containing the methyl esters.

NOTE 1: Boron/trifluoride is poisonous. It is not recommended that the analyst prepare the methanolic solution of boron trifluoride from methanol and boron trifluoride.

It is absolutely essential to prepare a boron trifluoride methanolic solution, proceed as follows:

Weigh a 2-litre flask containing 1 litre of methanol. Cool in a bath of iced water, keeping the flask in the bath bubble ‘BF_3 from a gas cylinder through a glass tube into the methanol until 125 g has been absorbed, doing this operation in the fume cupboard. Pass the stream of BF_3 through the glass tube before immersing the latter in the methanol and until it is removed, in order to prevent any liquid from returning to the gas expansion system. The gas should not give rise to white fumes by escaping too quickly from the flask. The reagent remains stable for 2 years if stored in a refrigerator.

13. Analysis by gas-liquid chromatography of fatty acid methyl esters

13.1 **Scope —** The method provides general guidance for the application of gas-liquid chromatography to determine qualitatively and quantitatively the composition of fatty acid methyl esters palm oil. It is identical with ISO 5508 ‘Animal and vegetable fats and oils – Analysis by gas-liquid chromatography of methyl esters of fatty acids’.

13.2 **Products Required**

13.2.1 *Carrier Gas* — Insert gas (nitrogen, helium, ………………. Thoroughly dried and with oxygen content less than 10- mg/kg.

13.2.2 *Auxiliary Gases*

13.2.2.1 *Hydrogen* (Purity 99.9 per cent) free from organic impurities.

13.2.2.2 *Air or oxygen* — Free from organic impurities.

13.2.3 *Reference Standards* — A mixture of methyl esters or the methyl esters of an oil of known composition, preferably similar to that of the fatty matter to be analysed.

13.3 **Apparatus —** The instructions given relate to the ordinary equipment used for gas liquid chromatography, employing a packed column and a flame-ionization detector. Any apparatus giving the efficiency and resolution defined in **19.4.1.2** is suitable.

13.3.1 *Gas Chromatography*

13.3.1.1 *Injection System* **—** The injection system shall have the least dead space possible. Unless materially impossible, it shall be capable of being heated to a maintaining the desired temperature to within 1ºC.

13.3.1.2 *Oven* **—** The oven shall be capable of heating the column to a temperature of at least 220 ºC and of maintaining the desired temperature to within 1 ºC.

If programmed heating is to be used, an apparatus with twin columns is recommended.

13.3.1.3 *Packed column*

13.3.1.3.1*Column* — The column shall be constructed of a material inert to the substances to be analysed (glass or stainless steel).

(*a*) *Length: 1 mm to 3 mm* — A relatively short column should be used when long-chain fatty acids (above 620) are present. When analyzing acids with 4 or 6 carbon atoms, it is recommended that a 2-m column be used.

(*b*) *Internal diameter* — 2 mm to 4 mm.

13.3.1.3.2 *Packing*

(a) *Support* — Acid-washed and silanized diatomaceous earth, or other suitable inert support with a narrow range of grain size (25 m), the average between the limits 125 m to 200 m), the average grain size being related to the internal diameter and length of the column.

(b) *Stationery phase* — Polyester type of polar liquid (for example, diethylene glycol polysuccinate, butanediol polysuccinate, ethyleneglycol polyadipate, etc) cyanasilicones or any other liquid. permitting the chromatographic separation required (*see* **14.4**). The stationery phase should amount to 5 per cent to 20 per cent of the packing. A polar stationery phase can be used for certain separations.

13.3.1.3.3 *Conditioning of the column* **—** With the column disconnected if possible, from the detector, gradually heat the oven to 185 ºC and pass a current of inert gas through the fresh prepared column at a rate of 20 mL/min to 60 mL/min for at least 16 h at this temperature, and for a further 2 h at 195 ºC

13.3.1.4 *Detector* — The detector should preferably be capable of being heated to a temperature above that of the column.

13.3.2 *Syringe* — Maximum capacity 10 mL, graduated in 0.1 mL.

13.3.3 *Recorder* — If the recorded curve is to be used to calculate the composition of the mixture analysed, an electronic recorder of high precision, compatible with the apparatus used, is required. The recorder shall have the following characteristics:

(*a*) Rate of response below 1.5 s, preferably 1 s; (The rate of response is the time taken for the recording pen to pass from 0 per cent to 90 per cent following the sudden introduction of a 100 per cent signal).

(*b*) *Width of the paper* — 25 cm minimum;

(*c*) *Paper speed* — Adjustable to values between 0.4 cm/min and 2.5 cm/min.

13.3.4 *Intergrator or Calculator (Optional)* — Rapid and accurate calculation can be performed with the help of an intergrator or calculator. This shall give a linear response with adequate sensitivity, and the correction for deviation of the base line shall be satisfactory.

13.4 **Procedure —** The operations described below relate to the use of a flame-ionization detector.

13.4.1 *Test Conditions*

13.4.1.1 *Selection of optimum operating conditions* — In selecting the test conditions, the following variables should be taken into account:

(*a*) Length and diameter of the column;

(*b*) Nature and amount of the stationery phase;

(*c*) Temperature of the column;

(*d*) Carrier gas flow;

(*e*) Resolution required;

(*f*) Size of the test portion, selected in such a way that the assembly of the detector and electrometer gives a linear response;

(g) Duration of analysis;

Generally, the following values will lead to the desired results, i.e. at least 2 000 theoretical plates for methyl stearate and its elution within about 15 min.

Internal Diameter of Column	*Carrier Gas-flow*
mm	mL/min
2	15 to 25
3	20 to 40
4	40 to 60

Concentration of Stationery Phase	*Temperature*
%	ºC
5	175
10	180
15	185
20	185

where, the apparatus allows it, the injector should be at a temperature of about 200 ºC and the detector at a temperature equal to or higher than that of the column.

As a rule, the ratio of the flow rate of the hydrogen supplied to the flame-iodization detector to that of the carrier gas varies from 1:2 to 1:1 depending on column diameter. The flow of oxygen is about 5 to 10 times that of the hydrogen.

13.4.1.2 *Determination of efficiency and resolution* — Carry out the analysis of mixture of methyl stearate and oleate in about equivalent proportions (for example methyl esters from cocoa butter).

Choose the size of the test portions, the temperature of column and the carrier gas flow so that the maximum of the methyl stearate peak is recorded about ¾ of the full scale (Figure. 1).

Calculate the number of theoretical plates, *n* (efficiency) using the formula:

$$n = \frac{16\ \mathrm{dR}^2}{w}$$

*a*nd the resolution *R*, using the formula:

$$R = \frac{2}{w + w}$$

where,

dR = the rentation distance, measured in millimetres, from the start to the maximum of the peak for methyl stearate;

w and w = the widths, in millimetres, of the peaks for methyl stearate and methyl oleate respectively, measured between the points of intersection of the tangents at the points of inflection of the curve with the base line;

d = the distance between the respective peak maximum for methyl stearate and methyl oleate.

Operating conditions to be selected are those, which will afford at least 200 theoretical plates for methyl stearate and a resolution of at least 1.25.

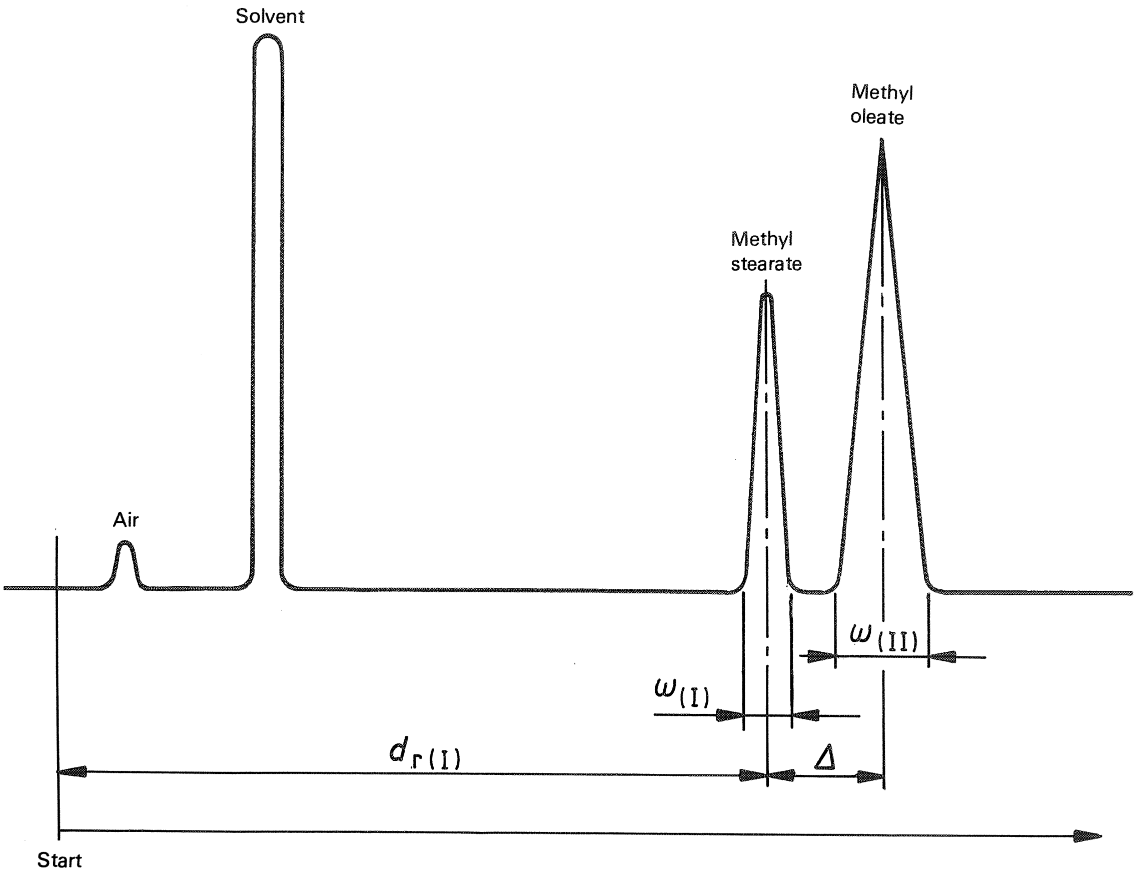

FIG. 1 — CHROMATOGRAM FOR DETERMINING NUMBER OF THEORETICAL PLATES (EFFICIENCY) AND RESOLUTION

13.4.2 *Test Portion* — Using the syringe (**19.3.2**) take 0.1 to 2 of the solution of methyl esters prepared. In the case of esters not in solution, prepare a solution, in heptane of chromatographic quality, of a solution, in heptane of chromatographic quality, of about 100 mg/mL and inject 0.1 to 1 of this solution.

If the analysis is for constituents present only in tea trace amounts, the size of the test portion may be increased (up to ten-fold).

13.4.3 *Analysis* **—** Generally, the operating conditions shall be those defined in **17.4.1.1**. Nevertheless, it is possible to work with a lower column temperature when the determination of fatty acids with fewer than 12 carbon atoms is required or at a higher temperature when determining fatty acids with more than 20 carbon atoms.

On occasion, it is possible to employ temperature programming in both the previous cases. For example, if the sample contains the methyl esters of fatty acids with fewer than 12 carbon atoms, inject the sample at 100 ºC (or at 50 ºC to 60 ºC if butyric acid is present) and immediately raise the temperature at 4 ºC to 8 ºC/min to the optimum. In certain cases, the two procedures can be combined. After the programmed heating, continue the elution at a constant temperature until all the components have been eluted. If the instrument does not work with programmed heating, work at two fixed temperatures between 100 ºC and 195 ºC.

If necessary, it is recommended that an analysis be carried out on two fixed phases with different polarities to verify the absence of masked peaks, for example for fish oils or in the case of the simultaneous presence C: and C: or C and C: conjugated.

13.5 Expression of Results

13.5.1 *Qualitative Analysis* — Analyse the reference standard mixture (*see* **19.2.3**), using the same operating conditions as those employed for the sample, and measure the rentation times or rentation distances for the constituent fatty acids. Construct on semi-logarithmic paper, for any degree of unsaturation, the graphs, showing the logarithm of the rentation time or distance as a function of the number or carbon atoms; in isothermal conditions, the graphs for straight-chain acids of the same degree of unsaturation should be straight lines. These lines should be approximately parallel.

Identify the peaks for the sample from these graphs, if necessary, to be interpolation.

It is necessary to avoid conditions such that 'masked peaks' exist. i.e. where the resolution is insufficient to separate two constituents.

13.5.2 *Quantitative Analysis — Determination of the Composition* — Apart from exceptional cases, use the internal normalization method, i.e. assume that the whole of the components of the sample are represented on the chromatogram, so that the total of the areas under the peaks represents 100 per cent of the constituents (total elution).

If the equipment includes an integrator, use the figures obtained therefrom. If not, determine the area under each peak by multiplying the height of the peak by its width at mid-height, and where necessary take into account the various attenuations used during the recording.

13.5.2.2 *Method of calculation and formula*

13.5.2.2.1 *General case* **—** Calculate the content of a given constituent, expressed as a percentage by mass of methyl esters, by determining the percentage represented by the area of the corresponding peak relative to the sum of the areas of all the peaks as follows:

Percentage by mass of component, expressed as methyl esters;

$$= \frac{A}{A} x 100$$

where,

A = the area under the peak corresponding to component;

A = the sum of the areas under all the peaks.

Give the results to one decimal place.

NOTE: In this general case, the results of the calculation based on relative areas is considered to represent a percentage by mass. For the cases in which this assumption is not allowed, *see* **19.5.2.2.2**.

13.5.2.2.2 *Use of correction factors* **—** In certain cases, particularly in the presence of fatty acids with fewer than 8 carbon atoms or of acids with secondary groups, when using thermal conductivity detectors or where the highest degree of accuracy is particularly required, correction factors should be used to convert the percentages of peak areas into mass percentage of the components,.

Determine the correction factors with the help of a chromatogram derived from the analysis of a reference mixture of methyl esters of known composition under operating conditions identical with those for the sample.

For this reference mixture:

Percentage by mass of component *i*

$$= \frac{m}{m} x\, 100$$

where,

m = the mass of component *i* in the reference mixture;

m = the total of the masses of the various components of the reference mixture.

From the chromatogram of the reference mixture, calculate:

Percentage (area/area) for component *i*

$$= \frac{\mathrm{Ai}}{\Sigma \mathrm{A}} \text{ x } 100$$

where,

A_i = the area under the peak corresponding to component *i*;

ΣA = the sum of the areas under all the peaks.

whence, correction factor:

$$Ki = \frac{m_i \ x \ \Sigma A}{A_i \ x \ \Sigma m}$$

commonly, the correction factors are made relative to *K*, so that the relative factors become:

$$K_{\mathrm{i}} = \frac{K^{\mathrm{i}}}{K_{C\,16}}$$

For the sample, the content of each component is given by:

Percentage by mass of component *i*, expressed as methyl esters,

$$= \frac{K_i \text{ x } A_i}{\Sigma\left(K_i \text{ x } A_i\right)} \text{x} 100$$

Give the results to one decimal place.

13.5.2.2.3 *Use of an internal standard* **—** In certain cases (notably the assay of c and c acids and the determination of the acids when all the fatty acids are not eluted) an internal standard should be used (c and c or c respectively) and the correction factor for the internal standard should be determined.

Percentage by mass of component *i*, expressed as methyl esters:

$$= \frac{\mathrm{M_S \text{ x } K_i \text{ x } A_i}}{\mathrm{m \text{ x } K_S \text{ x } A_S}} \text{x} 100$$

where,

m_s = the mass, in milligrammes, of the internal standards;

m = the mass in milligrammes, of the test portion;

K' = the correction factor for the internal standard (relative to K);

Ki' = the correction factor for component i (relative to K);

A_s = the area under the peak corresponding to the internal standard;

A_i = the area under the peak corresponding to component i.

Give the results to one decimal place.

13.5.2.3 *Repeatability* — The difference between the results of two determinations carried out on the same day by the same operator using the same apparatus for the same test material and for constituents present in excess of 5 per cent should not exceed a relative figure of 3 % of the determined value, with a maximum of 1 per cent absolute. For constituents present in smaller quantities, the difference should not exceed a value of 0.2 per cent absolute.

13.5.2.4 The difference between the results obtained in two different laboratories on the same test material for constituents present in excess of 5% should not exceed a relative figure of 10 per cent of the determined value, with a maximum of 3% absolute. For constituents present in value of 0.5 per cent absolute.

NOTE: By ‘test material’ is meant the oil, fat or fatty acid sample.

13.6 **Special Case —** Use of a catharometer detector (working on thermal conductivity changes). A gas-liquid chromatography employing a detector working on thermal conductivity changes (catharometer) may be used. If it is used, the conditions specified in **1.3** and **1.4** should be modified as follows:

(*a*) *Column Length* — 2 mm to 4 mm
Internal diameter — 4 mm

(*b*) *Support* — Grain size between 160 m and 200 m.

(*c*) *Amount of stationery phase* — 15 per cent to 25 per cent.

(*d*) *Carrier gas* — Helium or, failing this, hydrogen, with as low oxygen content as possible.

(*e*) *No auxiliary gases*.

(*f*) *Injector temperature* — From 40 ºC to 60 ºC above that of the column.

(*g*) *Column temperature* — 180 ºC to 200 ºC.

(*h*) *flow of carrier gas* — Usually between 60 mL/min and 80 mL/min.

(*i*) *For quantitative analysis* — Use the correction factors defined in **19.5.2.2.2**.

14. Determination of anisidine value

14.1 **Definition —** The anisidine value is defined by convention as 100 times the optical density measured in a 1-cm cell resulting from the reaction of 1 g of oil with 100 mL of the mixture of solvent and anisidine reagent.

The method determines the amount of aldehydes (principally 2 alkenals) in oils and fats.

14.2 **Principle —** Spectrophotometric measurement of the absorbance at 350 mm resulting from the reaction of the aldehydic compounds in an oil and p-anisidine in the presence of acetic acid.

14.3 Reagents

14.3.1 *ISO-Octane* (2,2,4-*Trimethylpentane) or n-hexane Optically Clear* — The absorbance of the solvent, measured in a 1-cm cell between 300 nm and 380 nm must be nil or nearly nil.

The commercial product can be freed from absorptive material by percolating it through a glass column (3 cm to 5 cm internal diameter, and 100-cm long) filled with silica gel.

14.3.2 *Glacial Acetic Acid* **—** The moisture content must be below 0.10 per cent/min. The presence of water tends to decrease the anisidine value since the reaction involves the formation of water. Check the moisture content by Karl Fischer titration. If it exceeds 0.1 per cent, discard the acetic acid.

14.3.3 *P-Anisidine* — (*see* Note 1) prepare a 0.25 per cent w/v) solution of the reagent in glacial acetic acid, (*see* **2**).

14.4 Apparatus

14.4.1 *Spectrophotometer — Suitable for use at 350 nm*.

14.4.2 *Glass cells — Of 1 cm light path*.

14.4.3 *Test-tubes — 10 mL with ground-glass* .

14.4.4 *Volumetric Flask* — 25 mL.

14.4.5 *Pipettes* — 5 mL and 1 mL.

14.4.6 Automatic pipettes or 1 mL pipette, with rubber bud to dispense the anisidine reagent.

14.5 **Preparation of sample** — Melt the sample at 60 ºC to 70 ºC and homogenize thoroughly before taking a test portion. The sample should be clear and dry. If not, it should be filtered and dried in a vacuum oven until the moisture content is below 0.1per cent.

14.6 **Procedure —** Weigh 0.3 g of the sample, to the nearest 0.001g into a volumetric flask. Dissolve and make up to volume with solvent.

Zero the instrument at 350 nm according to the manufacturer's instructions using the solvent.

Measure the absorbance (*A*) of the sample solution at 350 nm in the sample cell, using the reference cell filled with solvent as a blank. Pipette exactly 5 mL of the solvent into a second test tubeand exactly 5 mL of the solvent into a second test tube. By means of an automatic pipette, and exactly 1 mL of the p-anisidine reagent to each tube. Shake the tubes to homogenize the solution and reagent.

After exactly 10 minutes, measure the absorbance (A) at 350 nm of the solution in the first test-tube in the sample cell, using the solution from the second test-tube as a blank in the reference cell.

14.7 **Expression of Results**

$$AV = \frac{25\ x\left(1.2\ A_S - A_b\right)}{w}$$

where,

A_b = the absorbance of the sample solution after reaction with the p-anisidine reagent;

A_s = the absorbance of the sample solution;

w = the weight, in grammes, of the test portion.

NOTE 1: In storage, p-anisidine tends to darken as a result of oxidation. The commercial product, which is discoloured can be purified by one of the two methods:

(*a*) By molecular distillation using a small still with a cold finger under a pressure of 1 mm Hg.

(*b*) By recrystallization.

Dissolve 40 g of p-anisidine in 1 litre of water at 75 ºC. Add 2 g of sodium sulphate and 20 g activated carbon. Stir for five minutes and then filter through a fast filter paper. If carbon passes through, repeat the filtration using a double filter-paper.

Cool the filtrate to about 0 ºC and allow to stand for at least 4 h or preferably over-night. Filter off the crystals and wash with a small amount of water at a temperature around 0 ºC. Dry the crystals in a vaccum desiccator and then store in a brown glass bottle.

NOTE 2: Anisidine reagent solutions having an absorbance greater than 0.200 when measured in a 1.00 cm cell at 350 nm against the solvent as a blank should be discarded.

The solution should be handled with rubber gloves and the reagents dispensed with an automatic pipette or rubber bulb.

15. Methods for determination of refractive index

15.1 **Principle —** Measurement by means of a suitable refractometer, of the refractive index of a liquid sample at a constant temperature.

15.2 **Reagents —** All reagents shall be of recognized analytical quality.

15.2.1 *A-bromoaphthalene* — or

15.2.1 *Ethyl Laurate*

15.2.3 *Trichloethylene* — Or other suitable solvents such as hexane light petroleum, acetone, toluene, for cleaning the prism of the refractometer.

15.3 **Apparatus —** *Ordinary Laboratory Apparatus, and in Particular*:

15.3.1 *Refractormeter* — For example of the abbe tube, suitable for measurements of refractive index to within 0.0002 over the range n_D = 1, 7000.

15.3.2 *Light Source* — Sodium vapours lamp. White light can also be used if the refractometer is fitted with an echromatic compensation system.

15.3.3 *Glass Plate* — Of known refractive index.

15.3.4 *Water Bath*.

15.3.5 *Water Bath* — Thermostatically controlled, with a circulation pump, capable of being controlled to ± 0.1ºC

15.4 **Procedure**

15.4.1 *Preparation of the Test Sample* — The refractive index shall be determined on completely anhydrous and filtered fats and oils.

In the case of a solid sample, transfer the sample prepared to a suitable container and place it in the water bath (**20.3.4**), controlled at the temperature at which the measurements are to be made. Allow sufficient time for the temperature of the sample to stabilize.

15.4.2 *Calibration of the Instrument* — Verify the accuracy of the refractometer (**21.3.1**) by measuring the refractive index of the glass plate (**21.3.3**), ensuring optical contact with the prism my means of a drop or the a-bromonapthalene (**21.2.1**) or ethyl aurate (**21.2.2**)

15.4.3 *Determination* **—** Measure the reflective index of the sample at the following temperatures:

(*a*) 20 ºC for fats and oils, which are completely liquid at this temperature;

(*b*) 40 ºC for fats and oils, which are completely melted at this temperature;

(*c*) 60 ºC for fats which are not completely melted at 40 ºC

(*d*) 80 ºC or above for other fats and oils, for example, completely hardened fats, or waxes.

Maintain the temperature of the prism of the refractometer at the required constant value by circulation through its water from the water bath (**21.3.4**) controlled to the nearest 0.1ºC

Monitor the temperature of the water issuing from the refractometer using a suitable precision thermometer. Immediately before the measurement, lower the movable part of the prism to a horizontal position. Wipe the surface of the prism with a soft cloth and then with a few drops of the solvent. (**21.2.3**).

Carry out the measurements according to the operating instructions for the apparatus being used. Read the refractive index to the nearest 0.000 2 as an absolute value and record the temperature of the prism of the apparatus.

Immediately after the measurements, wipe the surface of the prism with a soft cloth and then with a piece of cotton wool moistened with a few drops of the solvent (**21.2.3**)

15.4.4 *Number of Measurements* — Measure the refractive index three times.

15.5 Expression of Results

15.5.1 Method of Calculation — If the difference between the measurement temperature, t_1 and the reference temperature *t* is less than 3 ºC, the refractive index n_Dt at the reference temperature *t* is given by the formula:

if $t_1 t$

$$n_D^t = n_D^{t_1} + (t_1 - t)\ \mathrm{F}$$

if $t_1 t$

$$n_D^t = n_D^{t_1} - (t_1 - t_1)\ \mathrm{F}$$

where,

t_1 = the measurement temperature, in °C;

tt = the reference temperature, in °C;

F = 0.000 35 at t = 20 ºC;

0.000 36 at t = 40 ºC and t = 60 ºC;

0.000 37 at t = 80 ºC

Take as the obtained result the arithmetic mean of the values obtained in the three measurements (**20.5.4**) provided that the requirement for repeatability (*see* **20.6.2**).

Report the results rounded to the fourth decimal place.

15.5.2 *Repeatability* **—** The difference between the values obtained in two measurements carried out simultaneously or in rapid succession by the same analyst shall not exceed 0.0002.

15.6 Test Report — The test report shall show the method used and the results obtained. It shall also mention any operating conditions not specified in this standard, as well as any incidents that may have influenced the results.

The test shall include all the information necessary for the complete identification of the sample.